THE ROLES OF *DANIO RERIO* NRF2 PARALOGS IN RESPONSE TO OXIDATIVE STRESS IN THE PANCREATIC β-CELL

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DEDICATION

I hope this work is a step towards curing Type 1 Diabetes. This step is for all the diabetic children in the world.

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Thank you to everyone who has helped and supported me in leading my first thesis research project.

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My family: parents Doszpoly Béla and Dr. Varga Katalin, and my sister, Doszpoly Éva

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THE ROLES OF *DANIO RERIO* NRF2 PARALOGS IN RESPONSE TO OXIDATIVE STRESS IN THE PANCREATIC β-CELL

Oxidative stress can disrupt cellular homeostasis, leading to cellular dysfunction and apoptosis. The Nrf2 transcription factor regulates the antioxidant response in cells by binding to antioxidant response elements (ARE) in DNA and activating genes of enzymes that combat oxidative stress. During the pathogenesis of diabetes mellitus (DM), β-cells are exposed to increased amounts of reactive oxygen species (ROS) that cause oxidative stress. Zebrafish (ZF) are excellent models for studying the dynamic mechanisms associated with DM pathogenesis, and we recently developed a ZF model of β-cell apoptosis caused by ROS. Two paralogs of Nrf2 have been identified in ZF, Nrf2a and Nrf2b, but their roles in pancreas development and/or β-cell survival are unknown. To investigate their roles, Nrf2a and Nrf2b antisense morpholinos (MO) were injected into Day 0 ZF embryos and analyzed over time. While Nrf2a MO showed no obvious phenotypes compared to WT, Nrf2b MO exhibited reduced pancreas size and islets with disrupted morphology. Ins:NTR Nrf2a MO showed reduced β-cell loss upon exposure to Metronidazole (MTZ) under generation of ROS compared to WT. Sequence analysis of ZF *nrf2b* in 3-day post-fertilization (dpf) embryos revealed a novel splice variant containing an additional exon that has not been described. Further investigation of Nrf2a and Nrf2b is likely to yield additional insights regarding the function and regulation of the NRF2-signaling pathway and their roles in β-cell protection under oxidative stress.

Amelia K Linnemann, PhD, Chair

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LIST OF ABBREVIATIONS

DM: Diabetes mellitus

T1DM: Type 1 Diabetes Mellitus

T2DM: Type 2 Diabetes Mellitus

ER: Endoplasmic Reticulum

ROS: Reactive oxygen species

NFE2L2/NRF2: Nuclear factor erythroid 2-related factor 2

Neh: NRF2f-ECH homology

CREB: cAMP Response Element Binding

ZF: zebrafish

MO: Morpholino

MTZ: Metronidazole

Ins: insulin

DNA: Deoxyribonucleic Acid

cDNA: complementary DNA

RNA: Ribonucleic Acid

ARE: Antioxidant response elements

dpf: Days post fertilization

bp: base pair

PCR: polymerase chain reaction

qPCR: quantitative polymerase chain reaction

KO: Knock-out

Ptf1a: Pancreas Associated Transcription Factor 1a

ELISA: enzyme-linked immunosorbent assay

BCA: Bicinchoninic Assay

ERSE: ER stress-response element

ERSRG: ER stress-response gene

INTRODUCTION

Diabetes Mellitus

Diabetes mellitus (DM) is a chronic disorder of glucose homeostasis that results in high concentrations of blood glucose throughout the body [1]. Current statistics show that DM remains the 7th leading cause of death in the United States, with a prevalence of 30.3 million in 2015. There are roughly 1.5 million new diagnoses each year and by 2040, approximately 642 million cases are predicted worldwide [2]. There are two major types of diabetes: Type 1 (T1DM) and Type 2 (T2DM). T1DM, also known as ""insulin-dependent diabetes", is an autoimmune disease triggered by an immune attack on the β-cells in the pancreatic islets of the endocrine pancreas, causing β-cell destruction and dysfunction, eventually leading to chronic glucose homeostatic disorder [3]. T2DM is defined as the body's inability to utilize the insulin it produces for metabolizing blood glucose. In T2DM, a key difference from T1DM is that, while there may still β-cell dysfunction, the most likely cause of diagnosis is insulin resistance caused by obesity [4]. Diabetes can lead to a number of additional complications and side effects, including blindness, higher risk of strokes and heart attacks, and neuropathy, due to complications from untreated high blood glucose concentrations [5].

DM physiology involves the destruction and dysfunction of β-cells in the pancreatic islets of the endocrine pancreas. β-cells are responsible for the synthesis, storage, and release of insulin for regulating glucose metabolism [6] (Fig. 1). Insulin is a peptide hormone that maintains normal blood glucose levels by facilitating cellular glucose uptake for proper homeostatic conditions [7][8]. β-cells are integral to the proper control of pancreatic physiology and are vital against the development of diabetes. In

T1DM or T2DM, β-cell mass and function are diminished, leading to insufficient insulin secretion and hyperglycemia [9]. Thus, survival of these cells is a key aspect of diabetes prevention. There are several biological mechanisms by which β-cells could be destroyed [10]. One of these is through endoplasmic reticulum (ER) stress that occurs with the generation of reactive oxygen species (ROS).

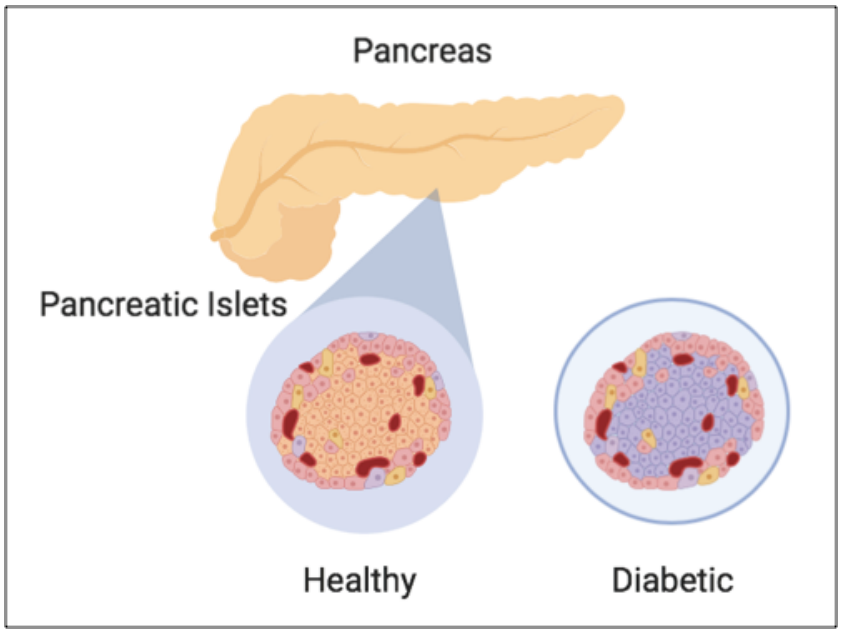


Figure 1. Diabetes physiology of the pancreas and β-cells. The pancreas consists of the endocrine and exocrine systems. The pancreatic islets, part of the endocrine system, are small groups of individual cells, including α, δ, and β-cells. α-cells release glucagon, while β-cells synthesize and secrete insulin. In a diabetic state, the β-cells are destroyed by the immune system and fail to secrete insulin into the blood.

Oxidative Stress

Oxidative stress is defined as a disturbance or imbalance between the production of oxidants, or ROS, from sources such as the mitochondria and ER, and antioxidant defenses in the cellular environment [11]. Such an imbalance in the system can lead to a range of impacts related to cellular homeostasis, including tissue damage, inflammation,

and damaged nucleotides that can further cause impaired protein production (Fig. 2). In DM, oxidative stress can have roles in the production of tissue damage in the β-cells that can lead to a number of negative impacts on cellular survival, including insulin synthesis and release upon high blood glucose presence [12].

Oxidative Stress in β-cells

Oxidative stress, in the context of β-cells, is an ideal model for T1DM for understanding the mechanisms of cell survival and death. β-cells are highly susceptible to oxidative stress [13]. During the pathogenesis of DM, β-cells are exposed to increased amounts of ROS that cause oxidative stress [14]. ROS are generated during the metabolism of glucose and serve as important signaling messengers to trigger insulin secretion and β-cell expansion in response to elevated levels of glucose. Chronic exposure to ROS under hyperglycemic conditions can lead to cellular damage, impaired glucose-stimulated insulin secretion, and eventually cell death [15].

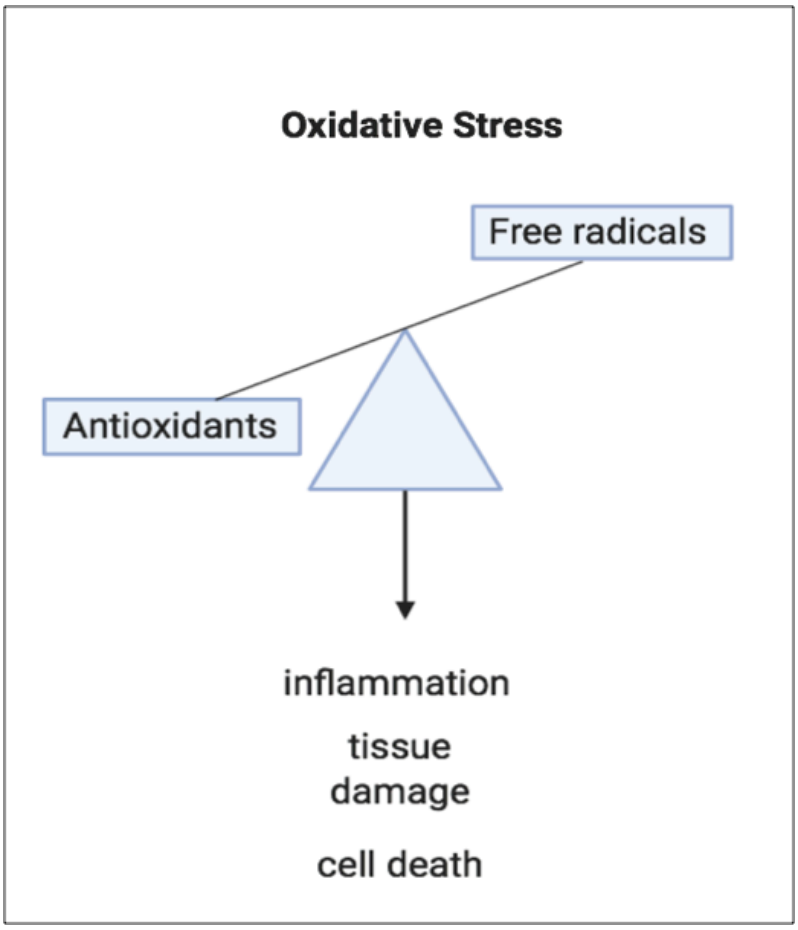


Figure 2. Oxidative stress and its effects. An imbalance between the antioxidants and free radicals in the cellular environment causes a disturbance in the system that can lead to inflammation, tissue and nuclear damage, and even cell death.

NRF2 and the Antioxidant Response

Balance is key for proper cellular functions and survival. Enzymes in cells are equipped to combat such shifts in the balance. These systems and molecules,

collectively, are known as the antioxidant response (ARE) factors. Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription factor and master regulator of the antioxidant response of cells [16]. NRF2 regulates the expression of AREs and target proteins and protect against oxidative damage, influencing a number of biological processes such as aerobic respiration, embryonic development, inflammation, and carcinogenesis [17-19].

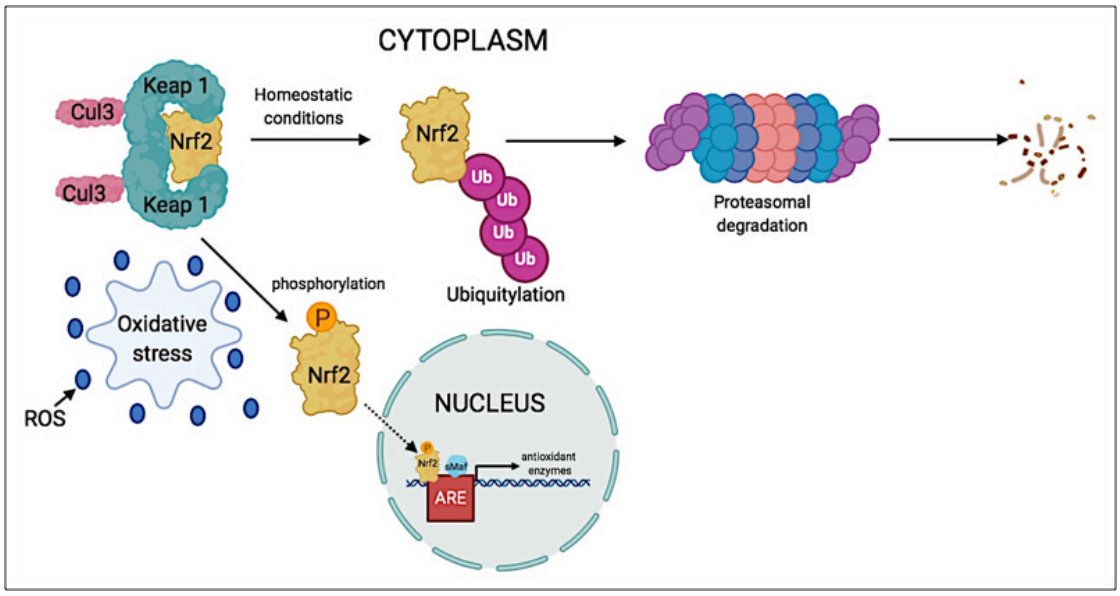


Figure 3. NRF2 activation mechanism. Under homeostatic conditions, the transcription factor NRF2 is present at low levels in the cytoplasm, bound to KEAP1 and Cul3, which are E3 ubiquitin ligases. Under non-oxidative stress conditions, NRF2 is ubiquitylated by KEAP1. Cul3 and is sent to proteasomal degradation. During oxidative stress conditions, NRF2 becomes phosphorylated and is released from KEAP1, allowing for NRF2 transport to the nucleus, where it binds to antioxidant response elements (AREs) in the promoters of genes involved in antioxidation.

NRF2 protein expressions in low, and Nrf2 is bound to its partner, Kelch like-ECH-associated protein 1 (KEAP1) and Cullin 3 (Cul3) in the cytoplasm [20]. However,

under oxidative stress, NRF2 is released from KEAP1 and translocates to the nucleus of the cell, where it binds to a DNA promoter and AREs located in proximity to promoters of target genes, activating gene transcription and their proteins. KEAP1 contains redox-sensitive cysteines that release NRF2 in the presence of oxidative stress and ROS [21] (Fig.3). Oxidative stress disrupts these cysteine residues and, therefore, disrupts the KEAP1-Cul3 ubiquitylation of NRF2. While NRF2 is in the nucleus, it combines with and forms heterodimers with small Maf proteins and the heterodimer complex binds to AREs to enhance initiation of transcription [22].

The NRF2 protein consists of six NRF2f-ECH homology (Neh) domains, originally assigned based upon regions identified as homologous between cross-species orthologs of NRF2 [23]. Each domain serves in key NRF2 functions. The Neh1 domain contains the DNA binding functions and serves to heterodimerize with small MAF proteins such as MAFF, MAFG, and MAFK [24]. Transactivation activities are promoted by the Neh3, Neh4, and Neh5. The Neh2 domain facilitates NRF2 binding to KEAP1 [21][25]. The Neh3 domain is also suggested to be involved in NRF2 protein stability [26]. The Neh4 and Neh5 domains also bind to cAMP Response Element Binding (CREB) proteins that are involved in intrinsic histone acetyltransferase activity [21]. Lastly, Neh6 domain is responsible in sensing redox and contains degrons involved in a redox-insensitive process of degradation of NRF2 [24][27]. Collectively, these domains ensure appropriate regulation of NRF2 transcriptional activities in response to ROS.

NRF2 in Diabetes Research

Recent studies have described mechanisms for NRF2 antioxidant regulation in pancreatic β-cells and the involvement of these processes in the pathogenesis of T1DM. Marasco et al., 2018 reported a novel finding that IL-6-driven ROS reduction is associated with an increase in NRF2 translocation to the mitochondria to decrease mitochondrial activity and stimulate mitophagy [28]. NRF2 regulation is likely to be important in the context of understanding β-cell protective mechanisms under oxidative stress conditions. IL-6-driven ROS reduction is associated with an increase in NRF2 translocation to the mitochondria to decrease mitochondrial activity and stimulate mitophagy. Additionally, studies have suggested that the NRF2 pathway is implicated in diabetic damage to the pancreas, heart, and skin, among other cell types and tissues in T2DM [29].

Zebrafish: an Important Developmental Model

Zebrafish models have been used for developmental studies due to their rapid embryonic stage development compared to other animal models [30]. Zebrafish are also an important vertebrate model for studying developmental toxicity with implications for understanding human embryonic development [31]. An advantage in the zebrafish genome is that there are often two copies of genes that are only present as a single copy in mammals. This allows for additional insights into the functions of the human counterpart [22].

The multiple gene copies present in the zebrafish genome are suggestive of the process of subfunction partitioning. A whole-genome duplication occurred during the divergence of fish and mammals [31] which gave rise to paralogous genes in the

zebrafish genome that are the duplicates of the single genes in mammals. Additionally, through this process, zebrafish paralogs have partitioned multiple functions of their mammalian orthologs [32][33].

nrf2a and nrf2b: Duplicate nrf2 genes in the Zebrafish Genome

Zebrafish possess six *nrf* –related genes, including two *nrf2* genes. The original zebrafish *nrf2* gene, designated as *nrf2a*, is positioned on chromosome 9 containing the single *hoxda* cluster [34]. Timme-Laragy et al., 2011 cloned a novel zebrafish *nrf2* paralog, *nrf2b* [34]. The human *NRF2* gene is located in a similar fashion adjacent to the HOXD cluster. In contrast, *nrf2b* is located on chromosome 6, near *miR-10d2* and other Hox-associated genes (*atp5g*, *lnp*, and *mtx2*). This region corresponds to that of the *hoxdb* gene cluster. Overall, the genomic mapping data demonstrates extensive conservation of *nrf* and *hox* genes in both zebrafish and human genomes. This conservation provides strong evidence that the zebrafish *nrf* genes are orthologs of the corresponding human *NRF* genes. We therefore propose that studying *nrf2a* and *nrf2b* genes in zebrafish will provide important insights into the regulation and functions of human NRF2 [34].

Initial sequence comparisons between *nrf2a* and *nrf2b* showed that *nrf2b* is lacking an exon corresponding to exon 3 in *nrf2a* (Fig. 4). Further, amino acid sequence alignments show that Nrf2b and Nrf2a are 25.1% identical overall, of which most of the identity match occurs in the conserved Neh domains. Comparisons of the Neh1 domain show thatNrf2a and Nrf2b share 73 and 41% identity, respectively. Neh1 is an important region, as it contains the CNC homology region and the basic-leucine zipper domains that

allow NRF2 to heterodimerize with small MAF proteins and bind to DNA. Further comparisons revealed that Nrf2b lacks the Neh4 transactivation. [35][36].

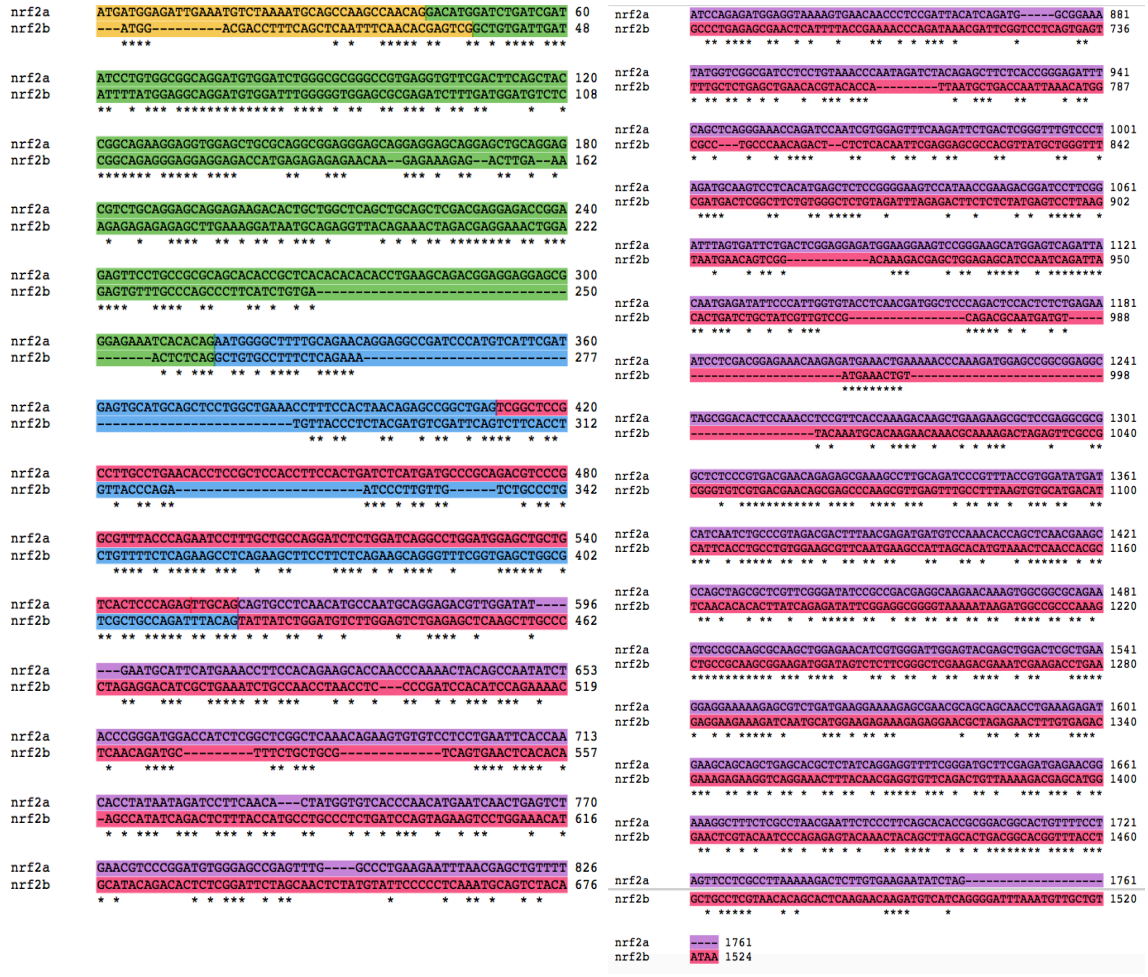


Figure 4. Coding sequence alignment of *nrf2a* and *nrf2b* with exons and homology using Clustal Omega. Exons 1 highlighted in yellow; 2 in green; 3 in blue; 4 in pink; and 5 in purple. Overall sequence identity is 51.86% between the two entries. *nrf2b* is lacking a region corresponding to exon 3 in *nrf2a*.

Timme-Laragy et al., 2011 also compared Neh domains of both ZF Nrf2a and Nrf2b to human NRF2 by analyzing the number of conserved amino acids over the total number in the specific domain. Their findings show that, overall, ZF Nrf2a has more

conserved amino acids in all Neh domains than Nrf2b, suggesting closer identity to human NRF2.

Antisense Morpholinos for Studying Gene Expression

Reverse genetics, including gene expression knockdown technologies, were used to study the functions of Nrf2a and Nrf2b in pancreatic β-cells under oxidative stress conditions. Antisense morpholinos (MO) are tools in molecular biology used for studying gene expression [37][38]. They are oligonucleotides with a methylenemorpholine ring backbone that bind to specific target sites within an mRNA sequence (Fig. 5). Morpholinos essentially inhibit mRNA translation by blocking other small molecules from accessing specific sequences on the mRNA [38]. The specificity of morpholinos is arguably favorable under proper experimental conditions. This is due in large to the 15 contiguous bases that the morpholino must bind to in order to ensure blocking a gene transcript, which constitutes sufficient sequence information for a selected gene of interest [39]. However, the possibility of undesirable off-target effects must be considered when administering morpholino dosages in experiments, as there has been evidence to suggest secondary sequence-specific p53 activation [40]. To avoid these effects, p53 inhibition could be considered applicable to these systems to suppress off-target effects caused by knockdown technologies such as morpholinos.

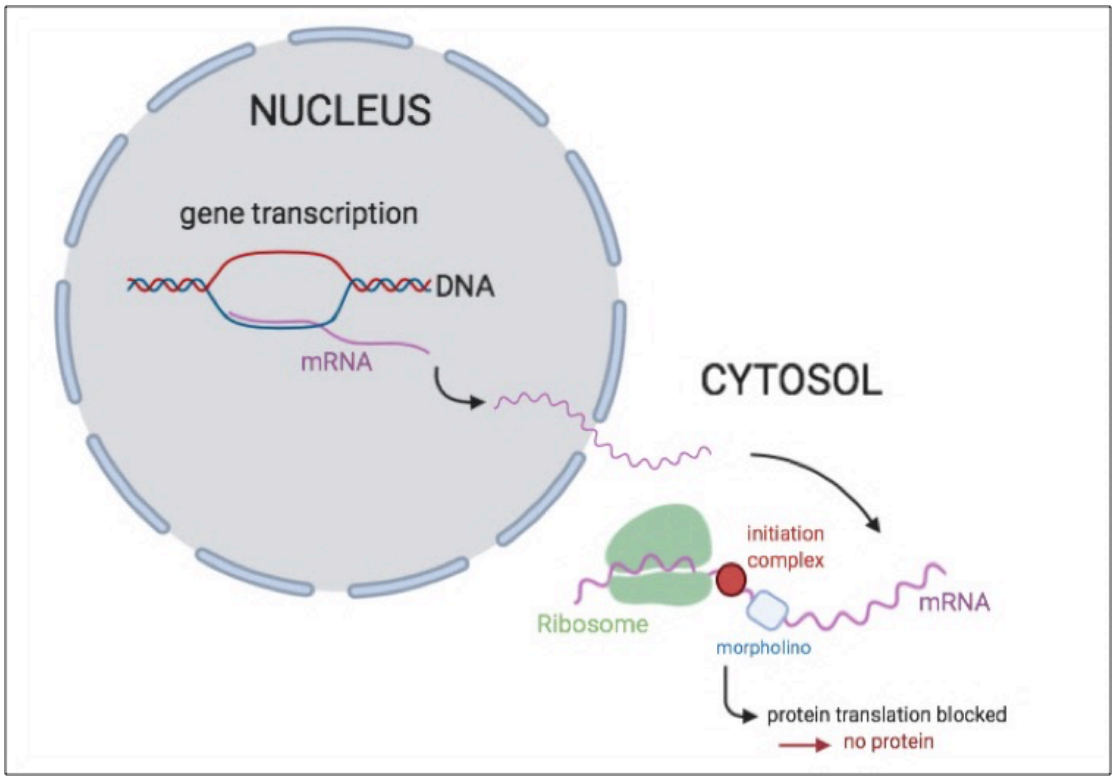


Figure 5. Schematic of the mechanisms by which morpholinos inhibit targeted gene expression. After gene transcription, the mRNA is exported out of the nucleus to bind to the ribosomal complex and begin protein translation. However, when a morpholino technique is used, the morpholino binds to the mRNA after the initiation complex and blocks mRNA translation, resulting in a reduced amount of expressed protein present.

Oxidative Stress in the Zebrafish Model

Having a vertebrate model to study ROS dynamics *in situ* and the factors involved in their mechanisms would provide insights into the discoveries and testing of therapeutics for a variety of metabolic diseases such as DM [41]. Kulkarni et al., 2018 proposed a model to study ROS generation in zebrafish in a β-cell-specific manner using a hybrid chemical genetic approach with the prodrug Metronidazole (MTZ) and a nitroreductase (NTR) system [42]. The model uses a transgenic NTR-expressing zebrafish line, *Tg(ins:Flag-NTR)*^{s950}, that expresses insulin promoter-driven NTR in β-cells exposed to specific concentrations of MTZ in a dose and time-dependent manner to study the generation of ROS throughout development. It ultimately showed strong evidence that the system effectively ablated β-cells under the insulin promoter to generate ROS and model T1DM pathogenesis [42].

The NTR-MTZ system has been implemented to efficiently ablate cells in a tissue-specific and temporally controllable manner [43]. The mechanism behind the technique is based on the *Escherichia coli* enzyme NTR to convert MTZ into a cytotoxic metabolite [44]. NTR is reduced by NADH or NADPH and causes MTZ to bind, become electrochemically reduced and then be converted into a DNA cross-linking agent responsible for ablating NTR-expressing cells [45-47]. When a fluorescent protein expressed under a tissue-specific promoter is treated with MTZ, it can induce rapid destruction of targeted cell population [42]. Through these interactions, MTZ is able to remain confined to the NTR-expressing cell; thus, cells near the targeted area are not affected, making the system specifically targeted. NTR-expressing cells can be monitored throughout the duration of the ablation through fluorescent microscopy.

Kulkarni et al.,2018 exposed INS-NTR ZF embryos to 7.5 mM MTZ for up to 24 hours, resulting in ablation of β-cells coincident with CellROX green staining indicated high levels of ROS [48][49]. ROS staining was observed in the β-cell nuclei within 1 hr of MTZ treatment, and gradually increased by almost 4-fold during the 24 hr treatment regimen. Further experiments with a range of MTZ concentrations concluded that a concentration as low as 2.5 mM was sufficient enough to induce β-cell death over the treatment duration [48]. The MTZ-NTR system is relevant to T1DM because ROS generation is specifically targeted to β-cell ablation and functional impair, much like the underlining physiology of T1DM pathogenesis. An advantage to this system is the ability to manipulate ROS generation under varied contexts due to the dependence of the dose of MTZ treatment, which can be easily administered for ZF experimentation methods.

HYPOTHESIS

Based on the roles of NRF2 in the regulation of oxidative stress, we hypothesized that Nrf2a and Nrf2b in zebrafish would regulate oxidative stress to protect the β-cells from ROS and have similar effects on mitigating ROS. Prior literature has shown that Nrf2a is more closely related to human NRF2, we further proposed that Nrf2a could be the more active, or preferred paralog of the two ZF Nrf2s, and be critical for β-cell resistance to ROS. To test the working hypothesis, the following experimental proposal was formed:

Investigative Questions

1. Are Nrf2 paralogs essential for exocrine or endocrine pancreas development?
2. Do Nrf2a and/or Nrf2b protect against oxidative stress in ZF β-cells?

Experiment 1. Knockdown expression of *nrf2a* and/or *nrf2b* by using antisense morpholinos. Using antisense morpholino technology to study protein function under knockdown conditions, we designed experiments that tested the functions of Nrf2a and Nrf2b under oxidative stress specific to the β-cell using the MTZ-NTR mechanism as done previously by Kulkarni et al., 2018.

Experiment 2. Determine phenotype of pancreas (overall pancreas and islet sizes) morphology and composition relative to overall embryonic development phenotypes using epifluorescence and confocal microscopy.

The investigation would be narrowed down to focus on the effects on pancreas development of the Nrf2a and Nrf2b MOs, as well as investigate Nrf2a MO and Nrf2b MO in the islets during oxidative stress induced by MTZ to model T1DM β-cell dysfunction.

Experiment 3. Inject morpholinos targeting Nrf2a and/or Nrf2b into insulin RFP, insulin NTR transgenic lines and determine response to MTZ in the presence and absence of Nrf2a and/or Nrf2b. Gene expression analysis will give insights to the underlying genetic regulations that occur in response to the Nrf2 paralog knockdown and will be analyzed through qPCR and gene specific primers in both MTZ treated and control embryos.

MATERIALS AND METHODS

Zebrafish Maintenance and Embryo Collection

Zebrafish were maintained at 28.5°C in a recirculating aquaculture system enclosed in a cabinet and subjected to 14-/10-hr light/dark cycle in accordance with institutional policies under IACUC oversight. Embryos were collected at spawning and maintained in a 28.5°C incubator in egg water-filled petri dishes. Transgenic zebrafish embryos were genotyped by epifluorescence at 48 hpf using a Leica M205FA dissecting microscope.

Morpholino Injections

Antisense morpholinos were purchased and stored in 4°C. Upon use, morpholinos were prepared with the injection mix at a 1:10 dilution along with Alexa Phenol Red dye. Injection mix was administered using a microinjector into 1-cell stage ZF embryos. Table 1 provides the sequences for both Nrf2a and Nrf2b MO.

Table 1. Sequences for Nrf2a and Nrf2b MO

Name	Sequence (5' → 3')
Nrf2a MO	CATTTCAATCTCCATCATGTCTCAG
Nrf2b MO	AGCTGAAAGGTCGTCCATGTCTTCC

Chemical Treatments

1-Phenyl-2-thiourea (PTU; Acros #207250250) supplementation at 0.003% was used to prevent pigmentation in all embryos after gastrulation stages. Metronidazole (MTZ)

(Sigma #095K093) solutions of 7.5mM were prepared in egg water (0.1% instant ocean salt, 0.0075% calcium sulfate) that was supplemented with PTU. Larvae were then paralyzed with 0.01% tricaine (Sigma #A-5040). 2 and 3dpf ZF embryos were fixed with 2.5% fixing solution (include ingredients and prep for this).

Antibody Staining, Immunofluorescence, and Confocal Microscopy Image Collection

Primary antibodies were stained at 1:100 ratio (insulin) and 1:200 (glucagon) for samples, with secondary antibodies at 1:500 each. After staining, embryos were mounted on slides in Vectashield (Vector Labs H-1000), mounted on glass-bottom petri dishes (Mattek #P35G-0-10-C) in 0.5% low melt agarose (Sigma #A9414). Imaging was performed on Zeiss LSM 700 confocal microscope at 40X zoom.

mRNA Measurements

RNA from ZF embryos was collected at 3 dpf and extracted using Trizol Phenol/Choloform extraction and Qiagen RNeasy kit with Ethanonl precipitation and DNAse treatments. CDNA was synthesized at 250ng RNA input using NAME OF KIT. qPCR analysis was measured with Quant Studio3 equipment on with iTAQ Master Mix (Applied Biosystems).

nrf2b Isoform Cloning and Sequencing

PJET 2.1 Cloning kit (Thermo Fisher Scientific #K1231) was used to clone the *nrf2b* PCR products into the vectors. Positive-stained bacterial E.Coli colonies were picked and plasmid DNA isolation was performed. Sequencing was performed by ACGT.

Primer Design for PCR

Primers for *nrf2a*, *nrf2b*, and *beta actin* for ZF were designed using NCBI Primer BLAST (NCBI References: *Nfe2l2a* (mRNA): NM_182889.1 *Nfe2l2b* (mRNA): NM_001257183.1) with exon-exon junctions and were validated on sample ZF DNA before experimentation. Table 2 provides the primer sequences for both *nrf2a* and *nrf2b*.

Table 2. Primer sequences for *nrf2a* and *nrf2b*

Name	**Sequence (5' → 3')**
nrf2a forward	TCACTCCCAGAGTTGCAGCAG
nrf2a reverse	CACTTCTGTTTGAGCCGAGC
nrf2b forward	ATCCCTTGTTGTCTGCCCTG
nrf2b reverse	TGGATCGGGGAGGTTAGGTT

PCR, qPCR and Gel Electrophoresis

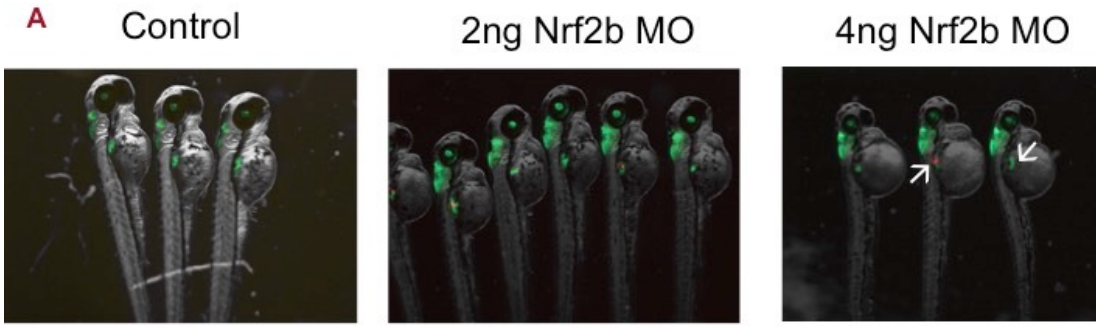
Q5 Hot Start High-Fidelity Polymerase (NEB M0493) was used for PCR reactions on 3 dpf ZF cDNA**.** Itaq Syber Green Mastermix kit was used for the qPCR reactions, performed in biological triplicates and technical duplicates. 2% and 1% agarose gels (Agarose in TEB) were made and loaded with samples run at 100V for 70 mins, stained with GelRed imaged under blue light.

RESULTS

Pancreatic phenotypes exhibit disrupted morphology and reduced pancreas size in Nrf2b MO ZF embryos

Our approach to study Nrf2 paralog functions under oxidative stress in the pancreatic β-cells began by looking at effects of Nrf2a and Nrf2b MO on the overall pancreas during embryonic development as compared to controls. The intent was to investigate and define pancreatic phenotypes generated by the effects of the morpholinos. Transgenic Ptf1a:GFP and Ins:red ZF were crossed, as these fish have fluorescently labeled exocrine and endocrine pancreas. Nrf2a and Nrf2b MO were separately administered at doses 2, 4, and 6ng at 1 cell stage. Embryos were allowed to develop until 3 dpf when they were imaged for pancreatic phenotypes. Fig. 6 (A) shows the images for Nrf2b MO and (B) the summary table of pancreatic phenotypes for Nrf2a MO and Nrf2b MO dosages. Overall, Nrf2a MO did not present any obvious pancreatic phenotypes in the range of MO dosages administered. However, Nrf2b MO exhibited disrupted morphology and reduced pancreas size at both the 2 and 4ng dosages. In addition to reduced pancreas size, there were also disrupted islets in some of the embryos as well. From these observations, a Nrf2b MO phenotype was identified and distinguished between the two Nrf2 paralogs.

A



B

Experimental Controls	2 ng MO dose	4 ng MO dose	6 ng MO dose
Nrf2b MO	Alive, not much difference to see yet	Alive, some possible phenotypes visible (smaller pancreas, enlarged islet)	All dead
Control	Alive, normal development	Alive, normal development	Alive, normal development

Figure 6. Pancreatic phenotype images of Nrf2a and Nrf2b MO. 3dpf *Ptf1a:GFP* and *Ins red* zebrafish embryos were injected with 0, 2, 4, and 6ng Nrf2b MO, respectively, and allowed to develop until 3dpf, when pancreatic phenotypes were analyzed. (A) Representative images of 3dpf embryos with no morpholino injection control samples (left) 2ng Nrf2b MO (center) and 4ng Nrf2b MO (left). (B) Summary table of Nrf2b MO pancreatic phenotypes and observations.

Nrf2a MO reduces β-cell loss after MTZ treatment

To further investigate Nrf2 paralog functions, I next analyzed β-cell survival under oxidative stress in MO vs. controls. My experimental design involved the use of the Ins:NTR-MTZ system for the generation of β-cell-specific ROS and a fluorescently labeled tag under the insulin promoter to allow visual examination of insulin positive cells. Fig. 7 describes the experimental timeline. Transgenic ZF (INS:NTR S950 X Ins:kaede) were crossed and embryos injected with 4ng Nrf2a MO at 1 cell stage. At 2dpf, embryos were fluorescently sorted for NTR and kaede positive transgenes. 7.5mM MTZ was administered to embryos for 24hrs after which embryos were immediately fixed with formaldehyde. Antibody staining and washes followed with confocal microscope imaging of islets. Fig. 8 shows representative confocal images and quantitative analysis of insulin positive cells for the experiment. For groups with no MTZ treatment, the presence of insulin positive β-cells seemed not to be affected in both control and Nrf2a MO groups. MTZ-treated control groups showed almost no presence of β-cells in the islet after treatment; however, Nrf2a MO MTZ treated group showed an increase of β-cell survival compared to controls. Quantitative analysis of the presence of insulin positive cells for each control group showed significance in Nrf2a MO groups for +/- MTZ treatment. Thus, contrary to our initial hypothesis, Nrf2a MO reduced β-cell loss after MTZ treatment.

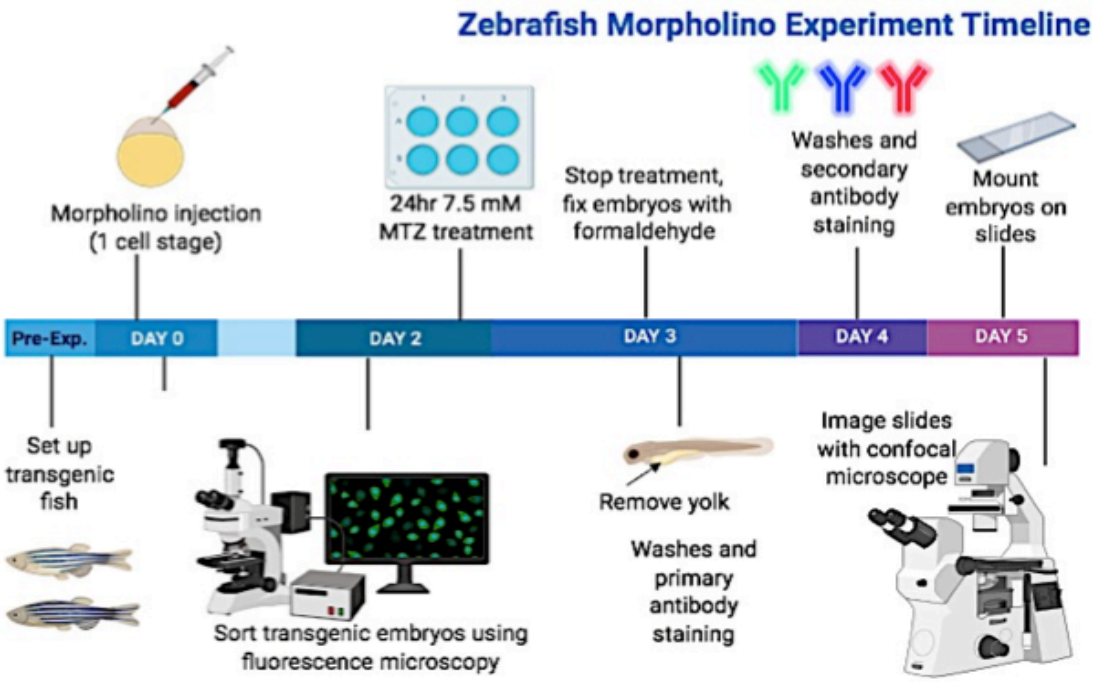


Figure 7. Zebrafish Nrf2a Morpholino MTZ experimental timeline. INS:NTR and WT transgenic ZF embryos (ins:kaede X S950 fish lines) were injected with 4ng Nrf2a MO at 1 cell stage. INS:NTR positive embryos were sorted at 2 dpf and administered 7.5 mM MTZ for 24hrs. Embryos were fixed with 3% formaldehyde and stained with primary and secondary antibodies for insulin and glucagon. Embryos were imaged with confocal microscope.

A **B.**

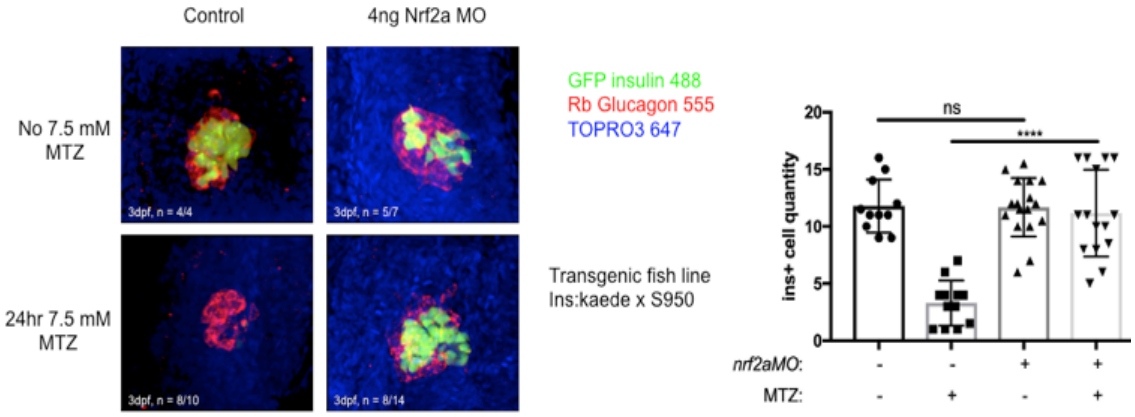


Figure 8. Nrf2a MO reduces β-cell loss after MTZ treatment. (A) Representative images of 3dpf embryos in the presence or absence of MTZ treatment for both controls and 4ng Nrf2a MO groups. No MTZ treatment resulted in β-cell survival in both controls and MO (top row); interestingly, while control group β-cells did not survive MTZ treatment (bottom left), the Nrf2a MO group showed β-cell survival and reduced loss after treatment (bottom right), suggesting a protective mechanism against ROS upon Nrf2a MO knockdown. (B) β-cell quantification for control and Nrf2a MO ZF in the presence of MTZ treatment or control MO. Number of β-cells after MTZ treatment between controls and Nrf2a MO are significant, averaging to around 3 to 12 cells, respectively.

Gene expression analysis of MTZ-treated Nrf2a MO and Control ZF suggests minute upregulation of nrf2b in Nrf2a MO embryos

Next, I addressed the mechanisms by which Nrf2a MO expression enhanced *β*-cell survival in response to MTZ treatment by targeting gene transcripts for *nrf2a* and *nrf2b* with qPCR. INS:NTR fish were crossed and injected with 4 ng of Nrf2a MO at 1 cell stage. At 2 dpf, embryos were sorted for INS:NTR positive transgenes with a fluorescence microscope. These embryos were then exposed to 7.5 mM MTZ for 3 or 24hrs, or vehicle, and RNA was isolated for each group followed by cDNA conversion. qPCR was then performed to measure mRNA expressed from the β-*actin*, *nrf2a*, and *nrf2b* genes. qPCR analysis for delta Ct values was calculated between *nrf2a* and *nrf2b* relative to *β-actin.* Values were normalized to controls for comparison purposes. The expression of *nrf2a* mRNA levels remained higher in the Nrf2a MO fish the longer the MTZ treatment, which was anticipated due to the actions of the morpholino. After 24hrs of MTZ, however, *nrf2a* expression decreases. In contrast, *nrf2b* expressions remained slightly higher after 3hr MTZ and nearly equal to control at 24hrs MTZ. A multi-way ANOVA performed on the data confirmed no significance (Fig. 9).

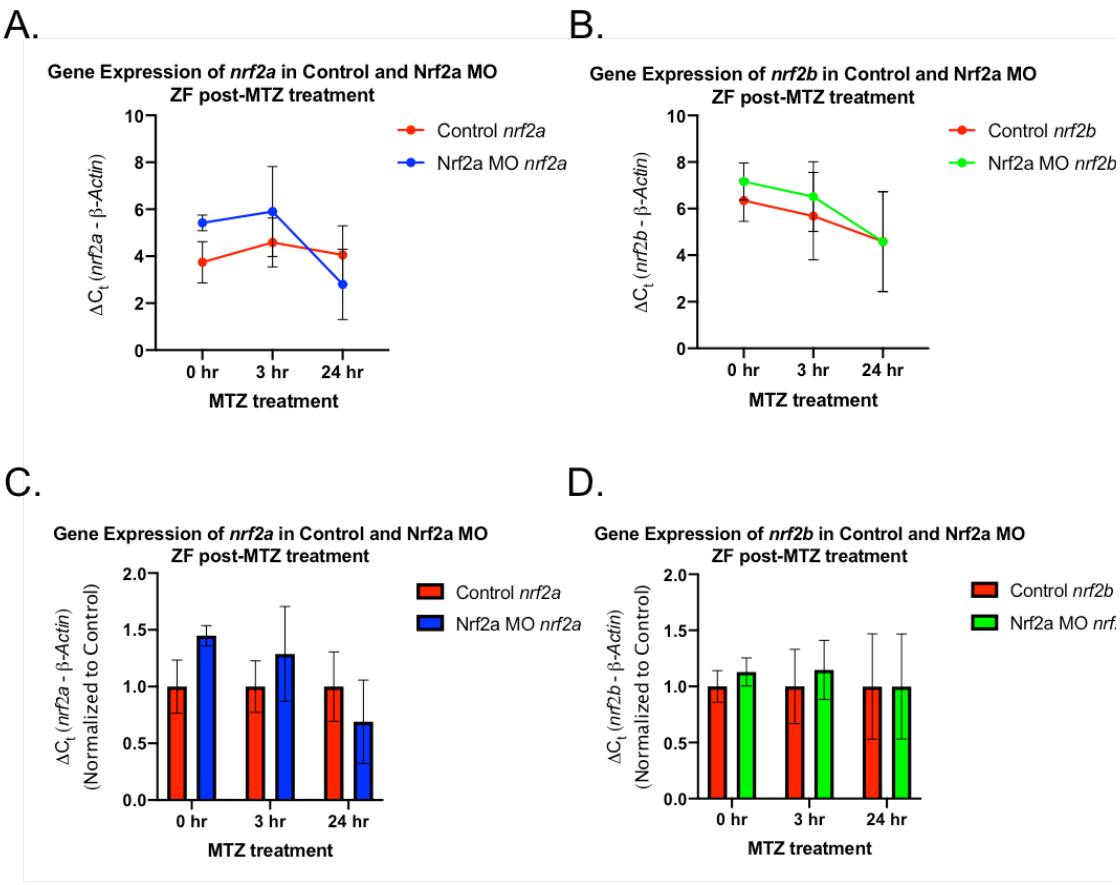


Figure 9. qPCR analysis of *nrf2a* and *nrf2b* gene expression in both Control and Nrf2a MO embryos after 0, 3, and 24 hr MTZ treatment. A-B: gene expression for *nrf2a* in Nrf2a MO and controls (A) and *nrf2b*. C-D: Data normalized to Control values for each time point. Multi-way ANOVA concluded no significance between the data. The *nrf2a* expression levels remained high in the Nrf2a MO the longer the MTZ treatment. After 24hrs of MTZ, however, *nrf2a* expression decreased. *nrf2b* expressions remained slightly elevated.

Sequence alignment of 3dpf ZF cDNA reveals novel splice variant of nrf2b

Initial PCR analysis was performed on 3dpf ZF cDNA to validate the designed primers for both *nrf2a* and *nrf2b*. Expected product sizes were 156bp and 186bp for *nrf2a* and *nrf2b*, respectively. Gel electrophoresis results showed both bands of products at the expected lengths; however, an additional band appeared in the *nrf2b* lane that was slightly greater in product size than the expected product (~200 bp). The new band appeared for each repetition of this procedure, eliminating the possibility of random product formation (Fig. 10).

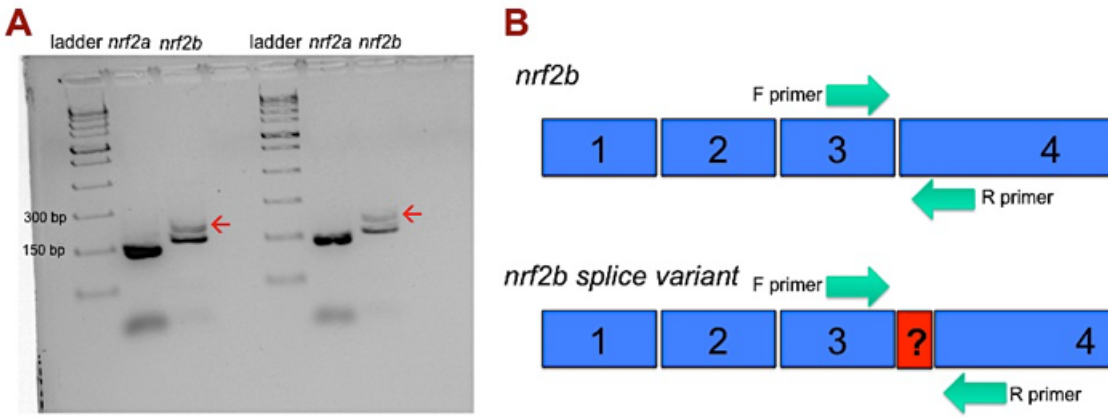


Figure 10. Sequence alignment of 3dpf ZF cDNA reveals novel splice variant of *nrf2b*. (A) Agarose gel electrophoresis showing PCR products of *nrf2a* (lane 2) and *nrf2b* (lane 3). Note the two products formed by *nrf2b* primers at lengths 186 bp (expected) and ~210 bp. (B) Proposed model of *nrf2b* splice variant. Sequencing analysis revealed an inserted product between exons 3 and 4 that corresponded to the change in product size in gel electrophoresis results.

DISCUSSION

Oxidative balance is key for cellular survival. We examined the master antioxidant regulator NRF2 paralogs, Nrf2a and Nrf2b, in the ZF model for investigating their roles in β-cells under oxidative stress for diabetes pathogenesis. The first experiment sought to develop a phenotype of the overall pancreas in both Nrf2a and Nrf2b ZF embryos as compared to controls, which resulted in a disrupted morphology in the Nrf2b morphants. Next, we examined the ZF islets under oxidative stress conditions in both control and Nrf2a MO embryos. β-cell specific oxidative stress was induced using the MTZ-NTR system as previously described by Kulkarni et al., 2018. Antibodies for both insulin and glucagon were used to visually represent the islet for confocal microscopy analysis. Results presented that while control MTZ treated embryos showed little to no insulin positive cells, Nrf2a morphants showed a significantly greater number of cells remaining after treatment, contrary to the initial hypothesis of β-cell death. After discovering that Nrf2a MO reduces the loss of β-cells under oxidative stress conditions, we then further analyzed the gene expression levels for *nrf2a* and *nrf2b* in these embryos and found that *nrf2b* gene expressions were slightly upregulated in the Nrf2a MO embryos after 24hrs MTZ as compared to controls.

A significant find from this study is that Nrf2a MO reduces β-cell loss after MTZ treatment. There are a couple of hypotheses that could explain this. The first hypothesis is that Nrf2b acts as a compensatory upregulator for Nrf2a under knockdown conditions. This would suggest an increase of the Nrf2b transcript in the Nrf2a MO during high levels of ROS from the MTZ treatment (1-6 hrs) to accommodate the decrease in protein production of Nrf2a from the mechanisms of the morpholino. The gene expression

analysis seems to indicate a slight increase in *nrf2b* levels in the Nrf2a MO at 3 and 24hr MTZ when compared to controls. This evidence could indicate a dynamic relationship between the Nrf2 paralogs during stressed cellular environments. An important note is that the morpholino essentially reduces gene expression for the whole-body organism. β-cell DNA represents a very small portion of the entire ZF genome, and detecting β-cell specific protein changes and gene regulation are an additional challenge for studies like these without direct protein quantification and DNA isolation.

It is also suggestive that the β-cells that survived the MTZ treatment are actually regenerated cells after ROS exposure. This phenomenon could be tested in ZF with the use of the ins:kaede-UV method, which uses a green-to-red photoconversion of kaede in β-cells after being exposed to UV light to indicate regenerated cells [50]. Applying this method to the study would allow further insights into the counterintuitive observation of how the β-cells survived the 24hr MTZ treatment in Nrf2a MO. Additional staining for ROS by CellROX would provide specifics on the amount of ROS, as well as the location within the cell to study ROS dynamics with Nrf2 paralog combat mechanisms simultaneously.

Another hypothesis is that the β-cells of the Nrf2a MO embryos survived the MTZ treatment due to a completely different method not directly involved in the Nrf2 paralog pathways. It has been described previously in literature that macrophages are recruited to injured β-cells upon stress signals. In the normal, lean state, islet macrophages promote islet development and maintenance of normal insulin secretion upon glucose stimulation [51]. It is possible that during oxidative stress conditions, and with the lack of a reliable Nrf2a protein to combat and stimulate the antioxidant response,

that these macrophages took over the protective role of the Nrf2 paralogs and promoted the survival of the β-cells captured in the images.

Fig. 11 shows a proposed model that describes possible mechanisms of the Nrf2a/Nrf2b dynamics and regulation in the β-cell under oxidative stress. This model is based on the results from both the confocal microscopy and qPCR gene expression analyses. At low levels of ROS in the β-cells, both Nrf2a and Nrf2b are upregulated and act together to combat ROS and suppress oxidative stress conditions. At higher levels of ROS, a number of events could differ. This includes: an additional increase in the Nrf2a levels to combat ROS, which could ultimately trigger stress-induced cellular apoptosis; and a compensatory upregulation of Nrf2b to combat ROS in the β-cell.

It is also important to consider similar protein isoform mechanisms in response to a stress signal that could provide additional insights to the Nrf2a/Nrf2b relationship dynamics. A study by Thuerauf et al., 2004 investigated the roles of the ATF ER transmembrane protein isoforms, ATF6α and ATF6β, in ER stress response gene induction. Their findings suggest that, while ER stress activates nuclear translocation and ER stress-response element (ERSE) binding for both ATF6 isoforms, they possess opposing roles in gene induction. ATF6β inhibited ATF6α-mediated GRP78 promoter activation, as well as TN-mediated GRP78 promoter activation that require ATF6α. The required levels of ATF6α and ATF6β for ER stress-response gene (ERSRG) induction also differ, providing evidence that ATF6β is a very poor activator of ERSRG induction as compared to ATF6α, and it represses ATF6α-mediated ERSRG induction. ATF6β may serve as a transcriptional repressor to regulate ATF6α-activated ERSRG induction during stress response [52].

As with most scientific studies, there are limitations to these experiments that could provide further insights into this research. One of the more important setbacks is the lack of reliable and functional antibodies for ZF Nrf2a and Nrf2b. Although Nrf2 antibodies have been previously used for fluorescence microscopy on zebrafish samples [53][54], they have not specifically been used on the pancreas and β-cells. Currently, there are no Nrf2a and Nrf2b antibodies available [55][56]. These would have been useful in the confocal microscopy analyses of the ZF islets under oxidative stress to visually see where Nrf2a and Nrf2b proteins are present and localize to under these conditions. These antibodies would also have been useful for protein quantification, especially in the Nrf2a morphants embryos, to quantitatively verify the specificity of the morpholino. Current methods of detecting and quantifying proteins include, but are not limited to, Western blots, chromatography, enzyme-linked immunosorbent assay (ELISA), Bradford and Bicinchoninic Assay (BCA), and mass spectrometry [57]. Implementing some of these techniques for this study would be important for further protein analyses.

Future experiments for this project are also suggestive as evident from the conclusions. It would be interesting to study Nrf2a knockout (KO) ZF embryos under oxidative stress conditions using the same Ins:NTR-MTZ system and experiments as done previously. Nrf2a KO ZF would not have any Nrf2a protein produced in the organism throughout development, and would represent a phenotype which the Nrf2 mechanism would be more heavily reliant on the Nrf2b protein. Nrf2b mechanisms could be more specifically measured in Nrf2a KO, as well as provide additional insights to Nrf2a roles in combating ROS.

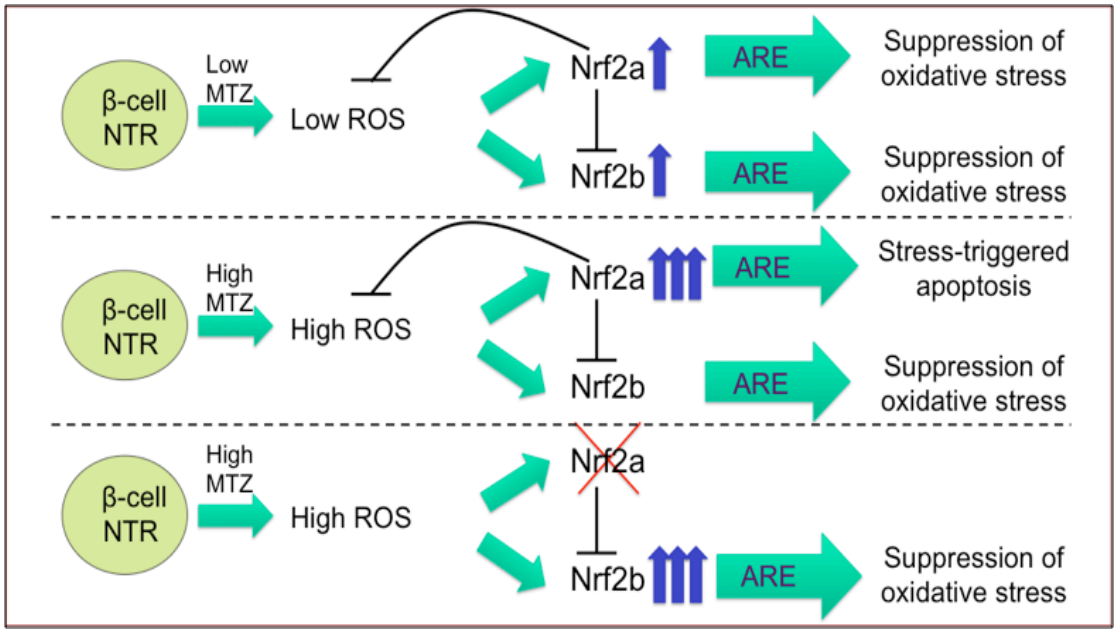


Figure 11. Proposed mechanisms of ZF Nrf2 paralog dynamics in *β*-cell targeted ROS generation. The top panel: low-dosage MTZ treatment triggers an increase in both Nrf2a and Nrf2b levels to suppress oxidative stress and ROS produced by the INS: NTR-MTZ chemical interactions. Middle panel: high-dosage MTZ treatment triggers an additional increase in Nrf2a levels, which in turn promote stress-triggered apoptosis of the cell. Nrf2b continues to combat ROS and suppress oxidative stress conditions. Bottom panel: high-dosage MTZ treatment, induced in Nrf2a knockdown or knockout conditions, triggers a compensatory upregulation of Nrf2b to combat oxidative stress in β-cells.

Another future experiment could be to investigate Nrf2b MO and perform the same ROS experiments and imaging analysis as for Nrf2a MO. The conclusions and proposed model, with regards to Nrf2b dynamics, could be tested out with Nrf2b MO and even knockout fish. Multiple fishlines can be created to feature Nrf2b knockouts. Similar to that of the ATF6 isoforms,

There could be some experiments performed with the different *nrf2b* splice variants to investigate if these transcribe any Nrf2b isoforms, and whether these have some special role in combating ROS in the β-cells. Perhaps there is a preferred isoform of Nrf2b that the cell uses most in the antioxidant response that requires a mechanism other than that of Nrf2a. There are several investigative questions that could arise with these hypotheses.

CONCLUSIONS AND SUMMARY

In summary, Diabetes remains a chronic metabolic condition that needs further understanding and insights into the biological aspects of the β-cell survival. During the pathogenesis of diabetes mellitus, β-cells are exposed to increased amounts of ROS that cause oxidative stress. Oxidative stress plays an essential role in disruption of cellular homeostasis and can further cause cellular dysfunction and potential death. The endogenous antioxidant response is the cell's combat mechanism against oxidative stress and prevents the proliferation of damage. The Nrf2 transcription factor regulates the antioxidant response in cells by binding to AREs in DNA and activating genes of enzymes that combat oxidative stress. ZF have two paralogs of Nrf2, Nrf2a and Nrf2b, but their roles in pancreas development and/or islets are unknown. With the use of antisense morpholino technology, we investigated pancreatic development, islet survival, and gene expression levels during oxidative stress conditions in Nrf2a and Nrf2b MO. We found that Nrf2a MO showed no obvious phenotypes compared to controls in pancreas development; however, Nrf2b MO embryos exhibited reduced pancreas size throughout development, and had islets with disrupted morphology. Ins:NTR tagged fish in Nrf2a MO showed a significant number of β-cell survival upon exposure to MTZ under ROS generation as compared to controls. Gene expression analysis of ZF *nrf2a* and *nrf2b* in MTZ-treated and control embryos revealed a slight increase in *nrf2b* expression in the morphants after 24hr MTZ. Sequence analysis of ZF *nrf2a* and *nrf2b* in 3dpf embryos revealed a novel splice variant of *nrf2b* containing an additional exon that has not yet been described. Sequence alignment with human and mouse Nrf2 variants revealed little to no significant similarities, suggesting specificity to ZF. Collectively,

these data suggest roles for Nrf2a and Nrf2b in the pancreas and islets that could provide insights into oxidative stress responses in diabetes pathogenesis. Further investigation of Nrf2a and Nrf2b is likely to yield additional insights regarding the function and regulation of the NRF2-signaling pathway and their roles in β-cell survival, and identify potential therapeutic targets for T1DM.

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CURRICULUM VITAE

Agnes Doszpoly

Education

M.S. Biochemistry and Molecular Biology, Indiana University-Purdue University Indianapolis (IUPUI), Indianapolis, IN June 2020

B.S. Biomedical Engineering, Purdue University, West Lafayette, IN December 2017

Research Experience

Graduate Researcher, Indiana University School of Medicine, Research Laboratory of Dr. Amelia K Linnemann, August 2018 – present

- Thesis title: "The Role(s) of *Danio rerio* Nrf2 Paralogs in Response to Oxidative Stress in the Pancreatic Beta Cell"

Undergraduate Researcher, Weldon School of Biomedical Engineering, Research Laboratory of Dr. Tamara Kinzer-Ursem, June 2016 – December 2017

- Conducted research about protein purification and affinity chromatography
- Assisted graduate researchers with their work
- Developed fundamental laboratory skills for protein engineering and professional research laboratory responsibilities

Undergraduate Biomedical Engineering Laboratory Courses, Weldon School of Biomedical Engineering, August 2014 - May 2016

- Developed laboratory skills in the fundamentals of biomolecules, biomaterials, bioinstrumentation, and biotransport
- Interacted with lab partners through weekly experiments and assignments
- Communicated ideas about project objectives for design experiments

Professional Presentations

Doszpoly A, Kulkarni AA, Conteh AM, Muralidharan C, Anderson RM, Linnemann AK *The Role(s) of Danio rerio Nrf2 Paralogs in Pancreatic Development and β-cell Regeneration under Oxidative Stress* (poster)

5th Annual Center for Diabetes and Metabolic Diseases Symposium, Indiana University School of Medicine, Indianapolis, IN July 2019

Doszpoly A, Kulkarni AA, Conteh AM, Muralidharan C, Anderson RM, Linnemann AK
The Role(s) of Danio rerio Nrf2 Paralogs in Pancreatic Development and β-cell Regeneration under Oxidative Stress (poster)
12th Annual Midwest Islet Club (MIC) Conference, University of Michigan, Ann Arbor, MI May 2019

Projects

Danio rerio Nrf2 Paralog Roles in Response to Oxidative Stress in Pancreatic Beta Cell, September 2018 - present

- Investigating antioxidant response factors Nrf2a and Nrf2b in *Danio rerio* for beta cell survival under oxidative stress in diabetes pathogenesis model
- Zebrafish care and embryo collection
- Morpholino technology for controlling gene transcription and silencing
- Fluorescence and confocal microscopy skills for interpreting images with antibody staining for potential phenotypes

NRF2 Mitochondrial Translocation upon IL-6 treatment for INS1 Cells Mitochondria Genome Expression, August 2018 - present

- Designed PCR primers for INS1 mitochondrial genome and analyzed gene expression upon IL-6 treatment from qPCR data
- Collaborated with teammates on findings with NRF2 mitochondria translocation and correlation of gene expression

LAMP DNA Amplification for Pathogen Detection for Portable Biosensor, August 2017-July 2018

- Designed LAMP primers for *Salmonella enterica* DNA detection and amplification for a portable biosensor using nanotechnology and bead particle diffusion
- Mentored undergraduate researcher on similar project with Dengue and Chikungunya viruses with related protein engineering techniques

Global Design of Assistive Technology Study Abroad Program, Ireland May 2017

- Engineered an assistive medical device for a wheelchair rain cover for patient in Dublin, Ireland with team

Balancing Assistive Device for Peripheral Neuropathy Senior Design Project, January 2016 - December 2016

- Collaborated with teammates on project on a balancing assistive device for peripheral neuropathy
- Researched and analyzed data for device components during prototyping period
- Organized reports, experiments, and team assignments throughout the scope of the project
- Developed strong project management skills
- Presented finalized work to fellow classmates, professors, and industry partners at ceremony

Biomolecular Engineering Design Project, November 2016 - December 2016

- Created a theoretical design project for developing a rapid, point-of-care (POC) diagnostic test for the Zika Virus with team of engineering students
- Communicated ideas amongst teammates during design aspects of the project
- Presented final project to classmates and professor

Physiological Modeling of the Human Body Design Projects, January 2016 - May 2016

- Engineered theoretical solutions to real-world medical problems involving modeling, interpreting, and generating data for physiological processes of the human body
- Collaborated with team to establish unique, original answers to projects through critical thinking and problem solving
- Prepared reports of findings and conclusions for each project to professor

Academic Awards

- Semester Honors from Purdue University December 2016, May 2017
- Valedictorian of International School of Columbus May 2013
- Science Olympiad State and Regional Championships individual and team award March 2010 - March 2013

Honor Societies

- National Society of Leadership and Success May 2018

Experience

Research Assistant, Indiana University School of Medicine, Wells Center for Pediatric Research, Center for Diabetes and Metabolic Diseases, Indianapolis, IN

- Project: NRF2 Translocates to Mitochondria upon IL-6 in INS1 cells

Research Assistant, Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN

- Project: LAMP DNA Amplification for Pathogen Detection for Portable Biosensor

Undergraduate Research Presenter for Weldon School of Biomedical Engineering
West Lafayette, IN

- Presented poster for undergraduate research experience to advisory board members
- Addressed questions of interested attendees
- Represented Kinzer-Ursem laboratory during session

BMES Professional Development Committee member, August 2014 - December 2014

- Organized professional networking events for biomedical engineering students
- Documented agendas for each committee session

Computer and Language Skills

Microsoft Office, MATLAB, C, Python, Hungarian and English fluency, Spanish proficiency